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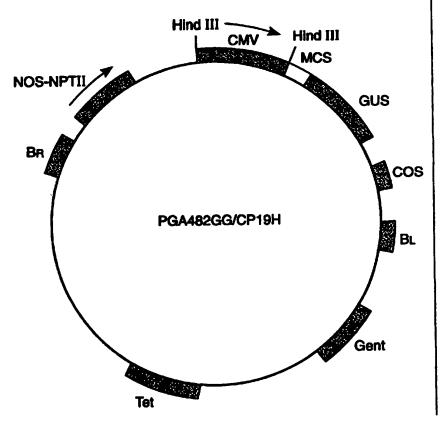
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With international search report.

(54) Title: TRANSGENIC PLANTS EXHIBITING HETEROLOGOUS VIRUS RESISTANCE

#### (57) Abstract

A transgenic plant transformed with a coat protein gene of cucumber mosaic virus strain C which confers resistance to heterologous virus challenge is provided.



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# TRANSGENIC PLANTS EXHIBITING HETEROLOGOUS VIRUS RESISTANCE

#### 5 Field of the Invention

This invention relates to a coat protein gene derived from cucumber mosaic virus strain C.

More specifically, the invention relates to the genetic engineering of plants and to a method for conferring viral resistance to a plant using an expression cassette encoding cucumber mosaic virus strain C coat protein.

#### Background of the Invention

Many agriculturally important crops are susceptible to infection by plant viruses, particularly cucumber mosaic virus, which can seriously damage a crop, reduce its economic value to the grower, and increase its cost to the consumer.

20 Attempts to control or prevent infection of a crop by a plant virus such as cucumber mosaic virus have been made, yet viral pathogens continue to be a significant problem in agriculture.

Scientists have recently developed means to 25 produce virus resistant plants using genetic engineering techniques. Such an approach is advantageous in that the genetic material which provides the protection is incorporated into the genome of the plant itself and can be passed on to its progeny. A host plant is resistant if it possesses the ability to suppress or retard the multiplication of a virus, or the development of pathogenic symptoms. "Resistant" is the opposite of "susceptible," and may be divided into: (1) high, (2) moderate, or (3) low 35 resistance, depending upon its effectiveness. Essentially, a resistant plant shows reduced or no symptom expr ssion, and virus multiplication within it is reduced or negligible. Several different typ s of host resistance to virus s are recognized. The host 40 may be resistant to: (1) establishment of infection,

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(2) virus multiplication, or (3) viral movement. Cucumber mosaic virus (CMV) is a singlestranded (+) RNA plant virus that has a functionally divided genome. The virus genome contains four RNA 5 species designated RNAs 1-4. RNAs 3 and 4 encode the coat protein which is a protein that surrounds the viral RNA and protects the viral RNA from being degraded. Only RNAs 1-3 are required for infectivity because the coat protein, which is encoded by RNA 4, is also encoded by RNA 3.

Several strains of cucumber mosaic virus have been classified using serology, host range, peptide mapping, nucleic acid hybridization, and sequencing analyses. These CMV strains can be divided into two groups, which are designated "WT" (also known as subgroup I) and "S" (also known as subgroup II). The S group consists of at least three members. WT group is known to contain at least 17 members.

Expression of the coat protein genes from 20 tobacco mosaic virus, alfalfa mosaic virus, cucumber mosaic virus, and potato virus X, among others, in transgenic plants has resulted in plants which are resistant to infection by the respective virus. Heterologous protection can also occur. For example, the expression of coat protein genes from watermelon 25 mosaic virus-2 or zucchini yellow mosaic virus in transgenic tobacco plants has been shown to confer protection against six other potyviruses: bean yellow mosaic virus, potato virus Y, pea mosaic virus, clover 30 yellow vein virus, pepper mottle virus, and tobacco etch virus. However, expression of a preselected coat protein gene does not reliably confer heterologous protection to a plant. For example, transgenic squash plants containing the CMV C coat protein gene, a 35 subgroup I virus, which have been shown to be resistant to the CMV C strain are not protected to the same degree against several highly virulent strains of CMV: CMV V27, CMV V33, and CMV V34 which are all

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invention provides a method of imparting multi-virus resistance to a plant which is susceptibl to viruses, comprising:

- (a) transforming cells of said susceptible plant with a chimeric recombinant DNA molecule comprising a promoter functional in cells of said plant and operably linked to a DNA sequence encoding a protein of a first class of virus which is capable of infecting said plant;
- 10 (b) regenerating said plant cells to provide a differentiated plant; and
  - (c) identifying a transformed plant which expresses the coding DNA sequence so as to render the plant resistant to infection by said first class of virus, wherein the plant is also rendered resistant to infection by at least one other class of virus to which said plant is susceptible.

Another embodiment of the present invention
20 provides a method for providing resistance to
infection by viruses in a susceptible <u>Cucurbitaceae</u>
plant which comprises:

- (a) transforming <u>Cucurbitaceae</u> plant cells with a DNA molecule encoding a protein from a first class of virus which is capable of infecting said <u>Cucurbitaceae</u> plant;
- (b) regenerating said plant cells to provide a differentiated plant; and
- (c) selecting a transformed <u>Cucurbitaceae</u> which is

  expressed so as to render the plant resistant to
  infection by said first class of said virus, and
  to at least one other class of said virus.

The present invention is exemplified by the insertion of a virus coat protein (cp) expression

35 cassette into a binary plasmid and subsequent characterization of the resulting plasmid. For example, CMV coat protein expression cassette can be placed in the binary plasmid pPRBN. Subsequently,

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binary plasmids harboring these expr ssion cass ttes are mobilized into Agrobacterium and employed to transfer the virus coat protein gen s into plants, such as members of the <u>Cucurbitaceae</u> family, along with the associated selectable marker and/or reporter genes.

As used herein, with respect to a DNA sequence or "gene", the term "isolated" is defined to mean that the sequence is either extracted from its context in 10 the viral genome by chemical means and purified and/or modified to the extent that it can be introduced into the present vectors in the appropriate orientation, i.e., sense or antisense. As used herein, the term "chimeric" is defined to mean the linkage of two or 15 more DNA sequences which are derived from different sources, strains or species, i.e., from bacteria and plants, or that two or more DNA sequences from the same species are linked in a way that does not occur Thus, the DNA sequences useful in the native genome. 20 in the present invention may be naturally-occurring, semi-synthetic or entirely synthetic. sequence may be linear or circular, i.e, may be located on an intact or linearized plasmid, such as the binary plasmids described below. As used herein, 25 the term "heterologous" is defined to mean not of the same virus class, i.e., a cucuomvirus, a potyvirus, a tabovirus, a comovirus, and a geminivirus are all As used herein, the different classes of viruses. term "expression" means transcription or transcription 30 followed by translation of a particular DNA molecule.

#### Brief Description of the Drawings

Fig. 1 depicts the binary plasmid pGA482GG/CP19H.

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#### Detailed Description of the Invention

Cucumber mosaic virus (CMV) is a singlestranded (+) RNA plant virus that has a functionally

divided genome. The virus genome contains four RNA speci s designated RNAs 1-4; 3389 nucl otid s (nt), 3035 nt, 2193 nt, and 1027 nt, respectively (P den et al., <u>Virol</u>., <u>53</u>, 487 (1973); Gould et al., <u>Eur. J.</u> 5 <u>Biochem.</u>, <u>126</u>, 217 (1982); Rezaian et al., <u>Fur. J.</u> Biochem., 143, 227 (1984); Rezaian et al., Eur. J. Biochem. 150, 331 (1985)). Only RNAs 1-3 are required for infectivity (Peden et al., <u>Virol.</u>, <u>53</u>, 487 (1973)) because the coat protein, which is encoded by RNA 4, is also encoded by RNA 3. Translations of CMV RNAs 10 yield a 95 kD polypeptide from RNA 1, a 94 kD polypeptide from RNA 2 (Gordon et al., Virol., 123, 284 (1983)), and two polypeptides from RNA 3: its 5' end encodes a 35 kD polypeptide, and its 3' end 15 encodes a 24.5 kD polypeptide (Gould et al., <u>Rur. J.</u> Biochem., 126, 217 (1982)). The 24.5 kD polypeptide is identical to that encoded by RNA 4 and is the coat protein.

Several strains of cucumber mosaic virus 20 have been classified using serology, host range, peptide mapping, nucleic acid hybridization, and sequencing. These CMV strains can be divided into two groups, which are designated "WT" (also known as subgroup I) and "S" (also known as subgroup II). 25 subgroup I includes CMV-C, CMV-V27, CMV-V33, CMV-V34, CMV-M, CMV-O, and CMV-Y while subgroup II includes CMV-Q, CMV-WL, and CMV-LS (Zaitlin et al., Virol., 201, 200 (1994)). Protection against a strain in one group does not necessarily provide protection against all strains in that group. For example, transgenic squash plants protected with coat protein genes from the CMV strain C are not protected against the CMV strains V27, V33, or V34. In addition, Zaitlin et al. (<u>Virol.</u>, <u>201</u>, 200 (1994)) report that tobacco plants transgenic for a CMV-FNY replicase gene show protection against challenge from subgroup I strains but show no protection against challenge from subgroup II challenges. Thus, the present invention is

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one of the part of the p
                                                                                                                                                                                                      CMV-C, for example, includes the DNA molecule having described by Onemada et al.
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                                                  (Samptook et al., Molecular Clouding: A laboration one of the NMA molecular clouding of the NMA 
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invention can include non-comp const
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expression of the CMV coat protein gen .

The CMV coat protein gene do s not contain the signals necessary for its expression once transferred and integrated into a plant genome.

5 Accordingly, a vector must be constructed to provide the regulatory sequences such that they will be functional upon inserting a desired gene. When the expression vector/insert construct is assembled, it is used to transform plant cells which are then used to regenerate plants. These transgenic plants carry the viral gene in the expression vector/insert construct. The gene is expressed in the plant and increased resistance to viral infection is conferred thereby.

When a viral gene expression cassette is 15 placed in a binary plasmid, and that plasmid transformed into a plant, the viral gene preferably exhibits substantially the same degree of efficacy to infection by at least two classes of virus when present in transgenic plants. More preferably, the 20 viral gene preferably exhibits substantially equal efficacy to infection by at least two classes of virus when present in transgenic plants. For example, if one examines numerous transgenic lines containing the viral gene expression cassette, a particular 25 transgenic line will be immune to infection by at least two viruses of different classes to substantially the same degree. Similarly, if a line exhibits a delay in symptom development to one virus, it will also exhibit a delay in symptom development to 30 at least one virus of a different class. Finally, if a line is susceptible to one of the viruses it will be susceptible at least one virus of a different class. Even with single gene constructs, one must test numerous transgenic plant lines to find one that displays the appropriate level of fficacy. 35 probability of finding a line with useful levels of expression can range from 10-50% (depending on the species involved). For further information refer to

PCT/US95/06263

Applicants' Assignees copending Patent Application
Serial No. \_\_\_\_\_\_ ntitl d "Transgenic Plants
Expressing DNA Constructs Containing a Plurality of
Genes to Impart Virus Resistance" filed on December
30, 1994, incorporated by reference herein.

Several different methods exist to isolate a viral gene. To do so, one having ordinary skill in the art can use information about the genomic organization of cucumoviruses to locate and isolate 10 the coat protein gene. The coat protein gene is located near the 3' end of RNA 3. Using methods well known in the art, a quantity of virus is grown and harvested. The viral RNA is then separated by gel electrophoresis. A cDNA library is created using the 15 viral RNA, by methods known to the art. The viral RNA is incubated with primers that hybridize to the viral RNA and reverse transcriptase, and a complementary DNA molecule is produced. A DNA complement of the complementary DNA molecule is produced and that 20 sequence represents a DNA copy (cDNA) of the original The DNA complement can be viral RNA molecule. produced in a manner that results in a single double stranded cDNA or polymerase chain reactions can be used to amplify the DNA encoding the cDNA with the use 25 of oligomer primers specific for viral sequences. These primers can include novel restriction sites used in subsequent cloning steps. Thus, a double stranded DNA molecule is generated which contains the sequence information of the viral RNA. These DNA molecules can 30 be cloned in E. coli plasmid vectors after the additions of restriction enzyme linker molecules by DNA ligase. The various fragments are inserted into cloning vectors, such as well-characterized plasmids, which are then used to transform E. coli and create a 35 cDNA library.

CMV coat protein genes from previously isolated strains can be used as hybridization probes to scre n the cDNA library to determine if any of the

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transformed bacteria contain DNA fragments with sequences coding for a CMV coat protein.

Alternatively, plasmids which harbor CMV coat protein sequences can be determined by restriction enzyme

5 digestion of plasmids in bacterial transformants. The cDNA inserts in any bacterial colonies which contain this region can be sequenced. The coat protein gene is present in its entirety in colonies which have sequences that extend 5' to the sequence which encodes the ATG start codon and sequences that extend 3' of the stop codon.

Alternatively, cDNA fragments can be inserted in the sense orientation into expression vectors. Antibodies against the coat protein can be used to screen the cDNA expression library and the gene can be isolated from colonies which express the protein.

In the present invention, the DNA molecules encoding the coat protein (CP) gene of the cucumber 20 mosaic virus strain C have been inserted into an expression cassette. This expression cassette can be placed into a vector that can be transmitted into plants, preferably a binary vector. The expression vectors contain the necessary genetic regulatory sequences for expression of an inserted gene. The coat protein gene is inserted such that those regulatory sequences are functional and the genes can be expressed when incorporated into a plant genome.

The segment of DNA referred to as the

30 promoter is responsible for the regulation of the
transcription of DNA into mRNA. A number of promoters
which function in plant cells are known in the art and
may be employed in the practice of the present
invention. These promoters may be obtained from a

35 variety of sources such as plants or plant virus s,
and may include but ar not limited to promoters
isolated from the caulimovirus group such as the
cauliflower mosaic virus 35S promoter (CaMV35S), the

enhanced cauliflower mosaic virus 35S promoter (enh CaMV35S), the figwort mosaic virus full-length transcript promot r (FMV35S), and the promoter isolated from the chlorophyll a/b binding protein.

5 Other useful promoters include promoters which are capable of expressing the potyvirus proteins in an inducible manner or in a tissue-specific manner in certain cell types in which the infection is known to occur. For example, the inducible promoters from phenylalanine ammonia lyase, chalcone synthase, hydroxyproline rich glycoprotein, extensin, pathogenesis-related proteins (e.g. PR-la), and woundinducible protease inhibitor from potato may be useful.

Preferred promoters for use in the present 15 viral gene expression cassettes include the constitutive promoters from CaMV, the Ti genes nopaline synthase (Bevan et al., Nucleic Acids Res. II, 369-385 (1983)) and octopine synthase (Depicker et 20 al., <u>J. Mol. Appl. Genet.</u>, <u>1</u>, 561-564 (1982)), and the bean storage protein gene phaseolin. The poly(A) addition signals from these genes are also suitable for use in the present cassettes. The particular promoter selected is preferably capable of causing 25 sufficient expression of the DNA coding sequences to which it is operably linked, to result in the production of amounts of the proteins or the RNAs effective to provide viral resistance, but not so much as to be detrimental to the cell in which they are 30 expressed. The promoters selected should be capable of functioning in tissues including but not limited to epidermal, vascular, and mesophyll tissues. actual choice of the promoter is not critical, as long as it has sufficient transcriptional activity to 35 accomplish the expression of the preselected proteins or RNAs, and subsequent conferral of viral resistance to the plants.

The non-translated leader sequence can be

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derived from any suitabl source and can be sp cifically modified to increase the translation of the mRNA. Th 5' non-translated r gion can be obtained from the promoter selected to express the gene, an unrelated promoter, the native leader sequence of the gene or coding region to be expressed, viral RNAs, suitable eucaryotic genes, or a synthetic gene sequence. The present invention is not limited

to the constructs presented in the following examples.

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The termination region or 3' non-translated region which is employed is one which will cause the termination of transcription and the addition of polyadenylated ribonucleotides to the 3' end of the transcribed mRNA sequence. The termination region may 15 be native with the promoter region, native with the structural gene, or may be derived from another source, and preferably include a terminator and a sequence coding for polyadenylation. Suitable 3' nontranslated regions of the chimeric plant gene include 20 but are not limited to: (1) the 3' transcribed, nontranslated regions containing the polyadenylation signal of Agrobacterium tumor-inducing (Ti) plasmid genes, such as the nopaline synthase (NOS) gene, and (2) plant genes like the soybean 7S storage protein 25 genes.

Selectable marker genes may be incorporated into the present expression cassettes and used to select for those cells or plants which have become transformed. The marker gene employed may express resistance to an antibiotic, such as kanamycin, gentamycin, G418, hygromycin, streptomycin, spectinomycin, tetracyline, chloramphenicol, and the like. Other markers could be employed in addition to or in the alternative, such as, for example, a gene coding for herbicide tolerance such as tol rance to glyphosat, sulfonylurea, phosphinothricin, or bromoxynil. Additional means of selection could

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include resistance to methotrexate, heavy metals, complementation providing prototrophy to an auxotrophic host, and the like. For example, see Table 1 of PCT WO/91/10725, cited above. The present invention also envisions replacing all of the virus-associated genes with an array of selectable marker genes.

The particular marker employed will be one which will allow for the selection of transformed cells as opposed to those cells which were not 10 transformed. Depending on the number of different host species one or more markers may be employed, where different conditions of selection would be useful to select the different host, and would be known to those of skill in the art. A screenable 15 marker or "reporter gene" such as the  $\beta$ -glucuronidase gene or luciferase gene may be used in place of, or with, a selectable marker. Cells transformed with this gene may be identified by the production of a 20 blue product on treatment with 5-bromo-4-chloro-3indoyl- $\beta$ -D-glucuronide (X-Gluc).

In developing the present expression construct, the various components of the expression construct such as the DNA sequences, linkers, or 25 fragments thereof will normally be inserted into a convenient cloning vector, such as a plasmid or phage, which is capable of replication in a bacterial host, such as E. coli. Numerous cloning vectors exist that have been described in the literature. After each 30 cloning, the cloning vector may be isolated and subjected to further manipulation, such as restriction, insertion of new fragments, ligation, deletion, resection, insertion, in vitro mutagenesis, addition of polylinker fragments, and the like, in order to provide a vector which will meet a particular 35 need.

For <u>Agrobacterium</u>-mediated transformation, the expression cassette will be included in a vector,

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Marobacterium tumefaciens, No. 5.258.300).
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Retrella, Nature, 303, 209 PCT WO/91/10725), al.

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present invention into plant certs.

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transformation using viruses or pollen, chemicals that increase the direct uptake of DNA (Paszkowski et al., <u>EMBO J.</u>, <u>3</u>, 2717 (1984)), microinjection (Crossway et al., <u>Mol. Gen. Genet.</u>, <u>20</u>2, 179 (1985)),

electroporation (Fromm et al., <u>Proc. Natl. Acad. Sci. USA</u>, <u>82</u>, 824 (1985)), or high-velocity microprojectiles (Klein et al., <u>Nature</u>, <u>327</u>, 70 (1987)).

The choice of plant tissue source or

10 cultured plant cells for transformation will depend on
the nature of the host plant and the transformation
protocol. Useful tissue sources include callus,
suspension culture cells, protoplasts, leaf segments,
stem segments, tassels, pollen, embryos, hypocotyls,

15 tuber segments, meristematic regions, and the like.
The tissue source is regenerable, in that it will
retain the ability to regenerate whole, fertile plants
following transformation.

The transformation is carried out under

conditions directed to the plant tissue of choice.

The plant cells or tissue are exposed to the DNA

carrying the present viral gene expression cassette

for an effective period of time. This may range from

a less-than-one-second pulse of electricity for

electroporation, to a two-to-three day co-cultivation

in the presence of plasmid-bearing Agrobacterium

cells. Buffers and media used will also vary with the

plant tissue source and transformation protocol. Many

transformation protocols employ a feeder layer of

suspended culture cells (tobacco or Black Mexican

Sweet Corn, for example) on the surface of solid media

plates, separated by a sterile filter paper disk from

the plant cells or tissues being transformed.

Following treatment with DNA, the plant

35 cells or tissue may be cultivated for varying lengths of time prior to selection, or may be immediat ly exposed to a selective agent such as those described hereinabove. Protocols involving exposure to

Agrobacterium will also includ an ag nt inhibitory to the growth of the Agrobacterium cells. Commonly used compounds are antibiotics such as cefotaxime and carbenicillin. The media used in the selection may be formulated to maintain transformed callus or suspension culture cells in an undifferentiated state, or to allow production of shoots from callus, leaf or stem segments, tuber disks, and the like.

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Cells or callus observed to be growing in

the presence of normally inhibitory concentrations of
the selective agents are presumed to be transformed
and may be subcultured several additional times on the
same medium to remove non-resistant sections. The
cells or calli can then be assayed for the presence of
the viral gene cassette, or may be subjected to known
plant regeneration protocols. In protocols involving
the direct production of shoots, those shoots
appearing on the selective media are presumed to be
transformed and may be excised and rooted, either on
selective medium suitable for the production of roots,
or by simply dipping the excised shoot in a rootinducing compound and directly planting it in
vermiculite.

In order to produce transgenic plants

25 exhibiting multi-viral resistance, a viral gene of the present invention must be taken up into the plant cell and stably integrated within the plant genome. Plant cells and tissues selected for their resistance to an inhibitory agent are presumed to have acquired the

30 selectable marker gene encoding this resistance during the transformation treatment. Since the marker gene is commonly linked to the viral genes, it can be assumed that the viral genes have similarly been acquired. Southern blot hybridization analysis using a probe specific to th viral genes can then be used to confirm that the foreign genes have been taken up and integrated into the genome of the plant c 11.

This technique may also give some indication of the

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number of copi s of the g ne that have been incorporated. Succ ssful transcription of the foreign qene into mRNA can likewise be assayed using Northern blot hybridization analysis of total cellular RNA 5 and/or cellular RNA that has been enriched in a polyadenylated region. mRNA molecules encompassed within the scope of the invention are those which contain viral specific sequences derived from the viral gene present in the transformed vector which are 10 of the same polarity to that of the viral genomic RNA such that they are capable of base pairing with viral specific RNA of the opposite polarity to that of viral genomic RNA under conditions described in Chapter 7 of Sambrook et al. (1989). mRNA molecules also 15 encompassed within the scope of the invention are those which contain viral specific sequences derived from the viral gene present in the transformed vector which are of the opposite polarity to that of the viral genomic RNA such that they are capable of base 20 pairing with viral genomic RNA under conditions described in Chapter 7 of Sambrook et al. (1989).

The presence of a viral gene can also be detected by immunological assays, such as the double-antibody sandwich assays described by Namba et al.,

25 Gene, 107, 181 (1991) as modified by Clark et al., J. Gen. Virol., 34, 475 (1979). See also, Namba et al., Phytopathology, 82, 940 (1992).

Virus resistance can be assayed via infectivity studies as generally disclosed by Namba et al., <u>ibid.</u>, wherein plants are scored as symptomatic when any inoculated leaf shows veinclearing, mosaic or necrotic symptoms.

It is understood that the invention is operable when either sense or anti-sense viral specific RNA is transcribed from the expression cassett s described above. That is, there is no specific molecular mechanism attributed to the desired phenotype and/or genotype exhibited by the transgenic

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Thus, protection against vital challenge can
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The alan undergrand rear areas and any one or any number of mechanisms.
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FURTHER INFORMATION, Serian Carial No.
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Expressing DNA Virus

Containing a plurality of Gener to Impart Virus

Containing a plurality of Gener to Impart Virus
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                                                                                                                                                                                                                                                                                    entitled "Transgenic plants Expressing DNA Virus

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it is necessary to breed line

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genetic control of viral resistance
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This requires crossing r sistant and s nsitive plants and studying the pattern of inheritance in segregating generations to ascertain wheth r the trait is expressed as dominant or recessive, the number of 5 genes involved, and any possible interaction between genes if more than one are required for expression. With respect to transgenic plants of the type disclosed herein, the transgenes exhibit dominant, single gene Mendelian behavior. This genetic analysis 10 can be part of the initial efforts to covert agronomically elite, yet sensitive lines to resistant lines. A conversion process (backcrossing) is carried out by crossing the original resistant line with a sensitive elite line and crossing the progeny back to the sensitive parent. The progeny from this cross 15 will segregate such that some plants carry the resistance gene(s) whereas some do not. Plants carrying the resistance gene(s) will be crossed again to the sensitive parent resulting in progeny which segregate for resistance and sensitivity once more. 20 This is repeated until the original sensitive parent has been converted to a resistant line, yet possesses all of the other important attributes originally found in the sensitive parent. A separate backcrossing 25 program is implemented for every sensitive elite line that is to be converted to a virus resistant line.

Subsequent to the backcrossing, the new resistant lines and the appropriate combinations of lines which make good commercial hybrids are evaluated for viral resistance, as well as for a battery of important agronomic traits. Resistant lines and hybrids are produced which are true to type of the original sensitive lines and hybrids. This requires evaluation under a range of environmental conditions under which the lin s or hybrids will be grown comm reially. Par ntal lines of hybrids that perform satisfactorily are increased and utiliz d for hybrid production using standard hybrid production practices.

21

The invention will be further described by
ref rence to th following detailed examples. Enzymes
were obtained from commercial sources and were used
according to the vendor's recommedations or other
variations known in the art. Other reagents, buffers,
etc., were obtained from commercial sources, such as
Sigma Chemical Co., St. Louis, MO, unless otherwise specified.

Most of the recombinant DNA methods employed in practicing the present invention are standard 10 procedures, well known to those skilled in the art, and described in detail in, or example, European Patent Application Publication Number 223,452, published November 29, 1986, which is incorporated herein by reference. General references containing 15 such standard techniques include the following: R. Wu, ed. (1979) Methods in Enzymology, Vol. 68; J.H. Miller (1972) Experiments in Molecular Genetics; J. Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual 2nd Ed.; D.M. Glover, ed. (1985) DNA 20 Cloning Vol. II; H.G. Polites and K.R. Marotti (1987) "A step-wise protocol for cDNA synthesis," Biotechniques 4; 514-520; S.B. Gelvin and R.A. Schilperoort, eds. Introduction, Expression, and Analysis of Gene Products in Plants, all of which are 25 incorporated by reference

## Example I. Squash Varieties With Multiple Virus Resistance.

#### A. <u>Cucumber Mosaic Virus</u>

The cloning, characterization and engineering of the CMV coat protein gene used in our experiments are described in H. Quemada et al., <u>J. Gen. Virol.</u>, <u>70</u>, 1065 (1989) and Slightom, <u>Gene</u>, <u>100</u>, 251 (1991).

10

#### B. <u>Binary Plasmid Vectors</u>

yielded pGA482GG.

The DNA which was transferred into the plant genomes was contained in binary plasmids (M. Bevan, Nucleic Acids Res., 11, 369 (1983)). The parent binary plasmid was pGA482, constructed by G. An, Plant Physiol., 81, 86 (1986). This vector contains the T-DNA border sequences from pTiT37, the selectable marker gene Nos-NPT II (which contains the plant-expressible nopaline gene promoter fused to the bacterial NPT II gene obtained from Tn5), a multiple cloning region, and the cohesive ends of phage lambda. Insertion of a bacterial gentamycin gene into the SalI site adjacent to the left T-DNA border of pGA482

The plant expressible CMV coat protein gene was cloned into the binary plasmid pGA482GG (for further inforamtion, see Applicants' Assignees copending Patent Application Serial No.

entitled "Transgenic Plants Expressing DNA Constructs

Containing a Plurality of Genes to Impart Virus Resistance" filed on December 30, 1994, incorporated by reference herein) to obtain pGA482GG/CP19H (Figure 1). Restriction enzyme site mapping showed that the CMV coat protein gene is oriented in the same

direction as the Nos-NPTII gene. Only the region between the two T-DNA border repeats will be transferred into the plant tissues.

After removal of seed coats the seeds were After removal of Beed coats, the Beeds, and a 20% and a Burraced Brerillized for 20-23 minutes in a 20% containing in the BOULTION OF BOOTUM NYPOCHLOVITE (Clorox) contains Disinfestation was tween 20 (200 ul/1000 mls.) tween 20 (200 ul/1000 mls.)

Times in sterile distilled in steriled in ster MO 36151035 Seeds were germinated in 150 x 25 mm culture water. Seeds were germinated in 150 x 25 mm culture in 150 x 25 mm culture and in 150 x 25 mm culture tunes containing 20 mls of 1/4 strength Murashige at 1/4 strength Mura After 5-7 days cotyledons were 0.8% Diffo Bacto Agar. After 3-1 days tips were imagenta the seedlings, car wasself imagenta removed from the seedlings, car wasself imagenta removed from the seedlings, and shoot tips were the seedlings, and shoot tips were excised and transferred to GAT was and and transferred to GAT. exclaed and transferred to GAT vessels (Magenta with a record and transferred to GAT wessels (Magenta with a record and transferred to GAT vessels (Magenta corp.) containing Agar. In a growth room at 25°C with a line around the around a growth room at 25°C with a growth room at 25°C w 0.8% Difco Bacto Agar. a photoperiod of 16 nours of light. (Phillips FAOCW)

Provided with both (Canara) plantain panantain panantain provided with provided with the provided with provided with both cool Liuorescent RAO-PR) lamps. cultures were incumated in a year of light.

a photoperiod of 16 hours of light. Leaf Pieces (0.5cm) were collected from in Leaf pieces (0.5cm) were collected timefactens in Agrobacterium tumefactens in Agrobacterium farron in 10.5cm) were collected timefactens in 10.5cm Agrobacterium tumefactens in 10.5cm Agrobacterium tumefacterium tumefact Vitro plants and Boaked in Agrobacterium tumeractens

Vitro plants and Boaked in Agrobacterium and transferred to 100

MC medium

Droth culture dienes containing an mis of MC medium

Agrobacterium tumeractens

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To 100

To 100 Droch culture (UD bUU U.1-U.2) and transferred to 100 more of MS medium

X 20 mm Petri dishes 2 mallirer 2 4 E. supplemented with 1.2 mg/liter 2.4.5- and 0.4 mg/liter plares trichlorophenoxyacetic (MAP) (MA-T) with 200 mM AG. a supplemented with 1.2 mg/liter 2.4.5. trichlorophenoxyacetic acid (4,4,3,2,1) and U.4,1 acid (MS-I) with 200 pm As.

benzylaminoacid (RAP) were incubated at 23°C. Arrex two three days lear negations are transferred onto MS-I medium containing and malliter reschanicallin Pieces were transferred onto 200 mg/liter cefotaxime and fare (MG-TA).

Soo mg/liter carbenicillin, and fare (MG-TA). 500 mg/liter carbenicillin, 200 mg/liter cefotaxime ten aufliter carbenicillin, 200 mg/liter [MS-IA]. Ma-TA medium sulfate [MS-IA]. Ma-TA medium and 150 mg/liter kanamycin auflate rranafarred to freeh MG-TA medium and 150 mg/liter kanamycin auflate rranafarred to freeh MG-TA medium and 150 mg/liter kanamycin auflate rranafarred to freeh MG-TA medium and 150 mg/liter kanamycin auflate rranafarred to freeh MG-TA medium and 150 mg/liter kanamycin auflate kanamy and low medium.

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medium containing 20 mg  $AgNO_3$ . Germinating embryos were subcultured to fresh medium until rooted shoots were obtained. Plantlets were transferred to soil for  $R_1$  seed production.

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#### D. Plant Analysis

Kanamycin resistant transformants were analyzed for the expression of the NPT II gene by ELISA using a commercially available ELISA kit (5-10 Prime 3-Prime, Boulder, CO). Polymerase chain reactions using the appropriate primers were conducted in order to amplify the NPT II gene (adjacent to the right border) and the coat protein gene closest to the left border. Some lines were further characterized using Southern Blot Analysis. Expression of the viral coat protein gene in putatively transformed plants was detected by ELISA utilizing alkaline phosphatase-conjugated antibodies according to the protocol of M.F. Clark et al., J. Gen. Virol., 34, 475 (1977).

20 Antisera to CMV-C, WMV-2, and ZYMV, were provided by D. Gonsalves (Cornell University, Geneva, New York).

The presence or absence of the T-DNA in the R<sub>1</sub> and subsequent generations was determined by ELISA tests for the selectable NPT II marker gene. PCR or Southern analysis was used to follow the inheritance in line ZW20 whose advance generations lacked the NPT II gene.

#### B. <u>Inoculation Procedure</u>

Segregating R<sub>1</sub> or R<sub>2</sub> progeny along with the appropriate control lines were germinated in the greenhouse. Prior to viral inoculation, cotyledon samples were collected for NPT II ELISA assays. Carborundum dusted cotyledons were mechanically inoculated on six-day-old seedlings with a 1x10<sup>-1</sup> wt/vol dilution of CMV strain C, ZYMV and WMV-2 which were propagated in Cucumis sativus, Cucurbita pepo and Phaseolus vulgaris, respectively. Plants were

inoculated with virus in the greenhous.

Approximately 7-10 days post inoculation, plants were transplanted into the field. In some trials non-inoculated control plants were included in order to monitor some spread of the virus by aphids. Data on symptomatic development were gathered prior to review of the NPT II ELISA results, so the scoring was done without knowledge of the transgenic status of the individual segregant being evaluated.

10 Plants were given a disease severity rating of 0-9 based on foliage symptoms (0 = non-symptomatic, 3 = symptoms on inoculated leave and/or very mild symptoms on new growth, 5 = moderate systemic spread 7 = severe systemic spread, 9 = severe systemic spread 15 and stunting). Fruits were also scored according to symptom severity (0 = non-symptomatic, 3 = mild green blotching of fruit. 5 = moderate discoloration. 7 = severe discoloration, 9 = fruit discoloration and distortion). Each line was then given a disease 20 rating for fruit and foliage which was an average of the individual plant ratings.

#### F. Field Trial Plot Design

Field trials were carried out under permits

issued by Animal and Plant Health Inspection Service
(APHIS) of the United States Department of Agriculture
(USDA). A design was employed in which each row
consisting of a transgenic line was paired with a row
containing its non-transgenic counterpart as a

control. Each row consisted of 15 plants, two feet
apart, with five feet between rows. Two to three
replications of each transgenic line were incorporated
in each test. Plots were surrounded by a minimum 30
foot border zone of non-transgenic squash plants in

order to reduce the flow of transgenic pollen out of
the trial site and to monitor for viral spread in the
field. Transgenic material incorporated into the test
included R<sub>1</sub> and R<sub>2</sub> progeny from self pollinated or

backcrossed R<sub>o</sub> yellow crookneck inbred lines. In some cases, a transgenic inbred lin was crossed to the appropriat nontransgenic inbred line in ord r to produce the transgenic versions of the commercial squash hybrids, Pavor or Dixie.

#### G. Results

As can be seen from the data summarized in Tables 1 and 2, below, all of the transgenic squash line 10 Paro-C-14-40 plants became infected when inoculated with ZYMV or WMV-2. However, as compared to the control plants, the disease ratings for both foliage and fruit were significantly less.

15

#### TABLE 1

Heterologous resistance in transgenic squash lines after inoculation with a 1/10 wt/vol dilution of WMV-2.

20

LINE	СР	Sympt #	omatic	Disease foliag fru		Ave # of fruit/p lant
Pavo-C-14 CMV-CP gene	+	13/13 15/15	100 100	6.8 7.0	2.5 6.1	1.3
Pavo control	+	30/30	100	7.0	7.0	1.5

25

TABLE 2

Heterologous resistance in transgenic squash lines after inoculation with a 1/10 wt/vol dilution of ZYMV.

LINB	CP	Sympt	omatic	Disease foliage fru:		Ave # of fruit/p lant
Pavo-C-14- 40 CMV-CP gene	+	13/13 15/15	100 100	6.3 6.7	6.7 8.7	1.7
Pavo control	+ -	28/28	100	7.2	8.7	2.2

10

15 All publications, patents and patent documents are incorporated by reference herein, as though individually incorporated by reference. The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

W 96/21032

#### WHAT IS CLAIMED IS:

- 1. A method of imparting multi-virus resistance to a plant which is
- 5 susceptible to viruses, comprising:
  - (a) transforming cells of said susceptible plant with a chimeric recombinant DNA molecule comprising a promoter functional in cells of said plant and operably linked to a DNA sequence encoding a protein isolated from a first class of a virus which is capable of infecting said plant;
  - (b) regenerating said plant cells to provide a differentiated plant; and
- 15 (c) identifying a transformed plant which
  expresses the coding DNA sequence so as to
  render the plant resistant to infection by
  said first class of virus, wherein the plant
  is also rendered resistant to infection by a
  second class of a virus.
  - The method of claim 1 wherein the first class of virus is a <u>cucumovirus</u> and the second class of virus is a <u>potyvirus</u>.

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10

3. The method of claim 2 wherein expression of the coding DNA sequence

imparts substantially equal levels of resistance to infection by each virus.

30

- The method of claim 1 wherein said DNA sequence encodes a virus coat protein.
- 35 5. The method of claim 2 wherein the expression of a coding DNA sequence imparts resistance to CMV infection.

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6. The method of claim 5 wherein the expression of said coding DNA

sequence further imparts resistanc to infection by WMV-2, ZYMV or to

5 infection by WMV-2 and ZYMV.

25

- 7. The method of claim 1 wherein the susceptible plant is a dicot.
- 10 8. The method of claim 7 wherein the susceptible plant is a member of the Cucurbitaceae family.
- 9. The method of claim 1 wherein the DNA molecule

  15 is part of a binary Ti

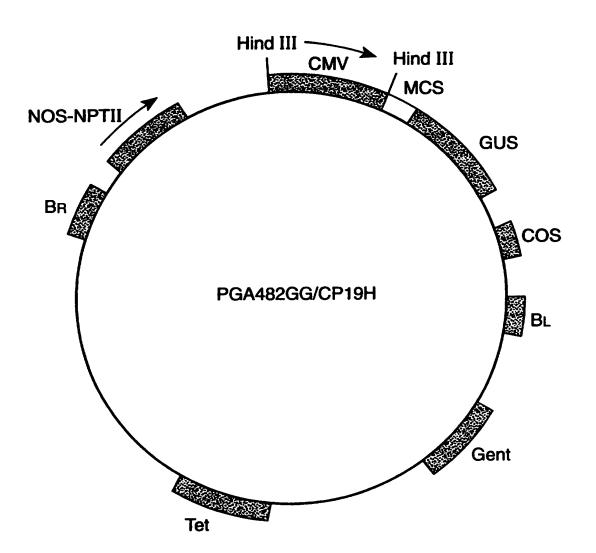
  plasmid and the plant cells are transformed by A.

  tumefaciens mediated

  transformation.
- 20 10. The method of claim 1 wherein the DNA sequence further comprises a selectable marker gene or a reporter gene that enables identification of said transformed plant.

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FIG. 1



## INTERNATIONAL SEARCH REPORT

Inter nal Application No PC1/US 95/06263

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According **	o International Patent Classification (IPC) or to both national classi	fication and IPC				
B. FIELDS	SEARCHED					
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X	PHYTOPATHOLOGY,		1,4,7,9, 10			
	vol. 79, 1989		10			
	pages 1284-1290, E.J. ANDERSON ET AL.; 'Transgen'	ic plants				
	that express the coat protein ger	nes of				
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	ther documents are listed in the continuation of box C.					
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### INTERNATIONAL SEARCH REPORT

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C.(Continu	BOOD) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages		Relevant to claim No.
A	PHYTOPATHOLOGY, vol. 82, 1992 pages 940-948, S. NAMBA ET AL.; 'Protection of transgenic plants expressing the coat protein gene of watermelon mosaic virus II or zucchini yellow mosaic virus against six potyviruses' see the abstract.		1
A	PHYTOPATHOLOGY, vol. 81, 1991 pages 794-802, H.D. QUEMADA ET AL.; 'Expression of coat protein gene from cucumber mosaic virus strain C in tobacco: protection against infections by CMV strains transmitted mechanically or by aphids' see the abstract.		1
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